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Free 25-Hydroxyvitamin D: Impact of Vitamin D Binding Protein Assays on Racial-Genotypic Associations

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Context: Total 25-hydroxyvitamin D (25OHD) is a marker of vitamin D status and is lower in African Americans than in whites. Whether this difference holds for free 25OHOD (f25OHD) is unclear, considering reported genetic-racial differences in vitamin D binding protein (DBP) used to calculate f25OHD.

Objectives: Our objective was to assess racial-geographic differences in f25OHD and to understand inconsistencies in racial associations with DBP and calculated f25OHD.

Design: This study used a cross-sectional design.

Setting: The general community in the United States, United Kingdom, and The Gambia were included in this study.

Participants: Men in Osteoporotic Fractures in Men and Medical Research Council studies (N = 1057) were included.

Exposures: Total 25OHD concentration, race, and DBP (*GC*) genotype exposures were included.

Outcome Measures: Directly measured f25OHD, DBP assessed by proteomics, monoclonal and polyclonal immunoassays, and calculated f25OHD were the outcome measures.

Results: Total 25OHD correlated strongly with directly measured f25OHD (Spearman r = 0.84). Measured by monoclonal assay, mean DBP in African-ancestry subjects was approximately 50% lower than in whites, whereas DBP measured by polyclonal DBP antibodies or proteomic methods was not lower in African-ancestry. Calculated f25OHD (using polyclonal DBP assays) correlated strongly with directly measured f25OHD (r = 0.80–0.83). Free 25OHD, measured or calculated from polyclonal DBP assays, reflected total 25OHD concentration irrespective of race and was lower in African Americans than in US whites.

Conclusions: Previously reported racial differences in DBP concentration are likely from monoclonal assay bias, as there was no racial difference in DBP concentration by other methods. This confirms the poor vitamin D status of many African-Americans and the utility of total 25OHD in assessing vitamin D in the general population. **(*J Clin Endocrinol Metab* 101: 2226–2234, 2016)**

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Abbreviations: BMI, body mass index; DBP, vitamin D binding protein; *GC*, group-specific component; mELISA, monoclonal ELISA; MRC, Medical Research Council; MrOS, Osteoporotic Fractures in Men; 25OHD, total 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; pRID, polyclonal radial immunodiffusion; pELISA, polyclonal ELISA; SRM, selected reaction monitoring.

Vitamin D is a precursor of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), with multiple effects in skeletal and extraskeletal tissues (1, 2). Total circulating 25-hydroxyvitamin D (25OHD) is used to diagnose vitamin D deficiency, but vitamin D bioavailability in extrarenal tissues is thought to depend on the small portion of 25OHD that is not bound to serum proteins. The concentration of bioavailable 25OHD (not bound to vitamin D binding protein [DBP]) or free 25OHD (not bound to DBP or albumin) may better reflect 25OHD function (3, 4). In some reports, stronger associations with free or bioavailable 25OHD than with total 25OHD were reported for serum calcium, PTH (5), bone mineral density (6), and vascular outcomes (7), suggesting that free or bioavailable 25OHD may provide a more clinically relevant measure. However, others have reported no improvement over total 25OHD (8). Nevertheless, the role of free 25OHD remains unresolved, as recently highlighted by the US Preventive Services Task Force (9).

Free 25OHD is calculated from the concentrations of total 25OHD, DBP, and albumin, with or without a factor accounting for DBP genotype-specific binding affinities (4, 6, 10, 11). DBP— or group-specific component (*GC*)— polymorphisms (rs4588 and rs7041) give rise to three major polymorphic isoforms of DBP (*GC*-1F, *GC*-1S, and *GC*-2), the frequencies of which differ globally, with *GC*-1F alleles more common in populations of African descent (12). Using DBP measured by a monoclonal ELISA, Powe et al reported that DBP was lower in African Americans than in US whites (13). As a result, calculated bioavailable 25OHD concentrations derived from those DBP measures were similar in whites and African Americans, a finding at variance with the lower mean circulating total 25OHD concentration consistently reported in African Americans (14, 15). The finding that low total 25OHD did not necessarily indicate low bioavailable 25OHD gained widespread attention in the medical and lay press (9, 16, 17) and may have important implications for nutritional supplementation policy. However, other publications did not report racial differences in DBP (18–21), and issues have been raised concerning the DBP measurements used by Powe et al (22, 23). Thus, racial differences in vitamin D availability and sufficiency remain uncertain.

To better understand these conflicting findings and investigate racial differences in total and free 25OHD, we conducted studies in cohorts based in the United States, United Kingdom, and The Gambia that included participants of African and European ancestry known to differ in *GC* genotype distribution. We characterized the molecular forms of circulating DBP through comparison of several DBP assays and proteomic analysis of DBP peptides. In addition, we compared total 25OHD to calculated 25OHD and directly measured free 25OHD and analyzed differences in concentrations by geographic region, race, and *GC* genotype.

Materials and Methods

Osteoporotic Fractures in Men (MrOS) cohort. The MrOS study enrolled 5994 participants (24). Recruitment occurred in six US communities, primarily through mass mailings. Participants were community-dwelling men older than 65 years of age. Informed consent was obtained, and the institutional review board at each site approved the study. From this cohort, 1020 men (101 African Americans and 919 non-Hispanic whites) had measurements of serum 25OHD and DBP and had *GC* genotype. Serum free 25OHD was measured in 194 randomly selected non-Hispanic white and 80 African-American participants (see Supplemental Materials and Methods). The mean age of participants was 74.8 (±6.2) years in whites and 71.1 (±5.4) in African Americans; and mean body mass index (BMI) was 26.7 (±4.6) kg/m^2 in whites and 28.8 (±4.7) in African Americans. As described in the following section, age and BMI adjustments were therefore employed to facilitate racial comparisons.

Medical Research Council (MRC) Gambian/UK cohort. Samples were from studies conducted at MRC Keneba, The Gambia, and MRC Human Nutrition Research , Cambridge, UK (19). Studies were approved by the joint Gambian Government-MRC Ethics Committee and the UK National Research Ethics Service, Cambridge Committee, respectively. Participants gave informed, written consent. All were healthy males, aged 25–39 years, and Gambians (n = 19) were of the Mandinka ethnic group; UK men (n = 18) were self-classified as white European. Plasma 25OHD, DBP, and directly measured free 25OHD concentrations were available for all participants and *GC* genotypes for a subset with sufficient DNA (Gambia, n = 17; UK, n = 12). There was no racial-geographic difference in mean age of participants, which was 29.3 (±4.4) years in the United Kingdom and 29.1 (±3.2) in The Gambia. Mean BMI was 22.6 (±2.3) kg/m^2 in the United Kingdom and 21.2 (±1.9) in The Gambia.

25OHD and 1,25(OH)$_2$D assays. In both cohorts, concentrations of 25OHD and $1,25(OH)_2D$ were measured with mass spectrometry (19, 25, 26).

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DBP assays. DBP was measured in all samples by polyclonal radial immunodiffusion (pRID) assay (18). In addition, two different polyclonal ELISA (pELISA) were used to measure DBP concentration: GenWay (GenWay Biotech) in MrOS and Immundiagnostik (Immundiagnostik AG) for the MRC study. Finally, DBP concentration was measured by monoclonal ELISA (mELISA; R&D Systems).

Genotyping. Two nonsynonymous *GC* single nucleotide polymorphisms were used to define *GC* diplotypes, rs4588 (Thr436Lys) and rs7041 (Asp432Glu).

Calculation of free 25OHD. Free 25OHD concentrations were calculated using published mathematical models (11).

Free 25OHD assay. Free concentrations of 25OHD were measured by ELISA (DIASource ImmunoAssays) (27, 28) at Future Diagnostics Solutions. This assay was validated by comparison with equilibrium dialysis at 37 C in 15 normal samples yielding a correlation of 0.83. The lower limit of detection was 1.9 pg/ml, and assay precision was less than 6% (29).

Proteomic analyses. Selected reaction monitoring (SRM) mass spectometry-based assays for DBP peptides encompassing the Thr436Lys and Asp432Glu substitutions, as well as separate peptides with no known sequence variation, were carried out in 120 MrOS serum samples selected to represent each of the six *GC* genotypes.

Further details of each method are in the Supplemental Materials and Methods.

Statistical analysis. Distributions of each 25OHD and DBP measure were examined for normality and outliers, and pairwise Spearman correlations were calculated in each cohort. Differences in total 25OHD, DBP, and free 25OHD concentrations between racial groups in each cohort were tested with unpaired Student's *t* tests and Wilcoxon rank-sum tests. *GC* genotype differences in mean total 25OHD, DBP, DBP peptide abundance, and free 25OHD were tested by ANOVA in each cohort separately and were followed by post hoc pairwise tests between genotypes. Linear regression was used to test whether associations with race in each cohort persisted after age and BMI adjustment. The model R^2 for each cohort was used to quantify the proportion of variance in DBP concentration that *GC* genotype contributed for each DBP assay. Analyses were performed in SAS 9.4 (SAS Institute Inc) and Stata 12 (StataCorp).

Results

Total circulating 25OHD concentrations varied by race and geography. UK whites had the lowest mean levels, and the mean total 25OHD was 56% higher in US whites than in African Americans and 137% higher in Gambians than in UK whites (Figure 1). There were similar differences in $1,25(OH)_2D$ concentrations, albeit of smaller magnitude (11% higher in US whites than African Americans and 67% higher in Gambians than UK whites). Mean DBP concentrations were minimally different across all groups using polyclonal assays, but the mean DBP concentration measured by mELISA was 54% lower in African Americans than in US whites and 52% lower in Gambians than in UK whites (Figure 1). Total 25OHD concentrations were weakly correlated with DBP, regardless of assay type (all Spearman $r \leq 0.28$).

Directly measured free 25OHD concentrations were strongly correlated with total 25OHD in all racial/geographic groups (Figure 2A; from $r = 0.71$ in UK whites to $r = 0.83$ in both groups of African descent, Supplemental Table 1). Similarly, calculated free 25OHD concentrations derived from polyclonal measures of DBP were highly correlated with total 25OHD (Figure 2B; $r = 0.96$ for pRID calculation, and $r = 0.93$ for pELISA calculation Supplemental Table 1). Concentrations of free 25OHD calculated from DBP measured by mELISA were less strongly correlated with total 25OHD or directly measured free 25OHD concentrations (Figure 2C, Supplemental Table 1). Free 25OHD concentrations calculated using a *GC*-genotype specific affinity generally had lower correlations with concentrations of total 25OHD or measured free 25OHD than that calculated using a constant affinity for all *GC* isoforms, although correlations were higher for mELISA in some groups (Supplemental Table 2).

The mean free 25OHD concentration was between 0.02% and 0.09% of the total 25OHD mean in all groups. Racial differences in free 25OHD reflected racial and country differences in total 25OHD when free 25OHD was directly measured or calculated based on polyclonal DBP concentrations; US whites and Gambians had higher free 25OHD than African Americans and UK whites, respectively (Figure 1). However, when free 25OHD was calculated with mELISA DBP, African Americans had a higher mean concentration than US whites (Figure 1).

As expected, there were marked racial differences in the distribution of *GC* genotypes. Nearly all (>97%) African Americans and all Gambians had a *GC*-1F allele (1F1F, 1F1S, and 1F2 genotypes), whereas in whites the majority had no 1F allele, and the predominant genotypes were 1S1S and 1S2 (Figure 3). There were striking differences in how DBP assay results were associated with *GC* genotypes. DBP concentrations measured by mELISA were strongly associated with *GC* genotypes (Figure 4). *GC* genotype accounted for 83% of the variation in mELISA DBP. Mean DBP concentrations in those with *GC*-1F1F and *GC*-1F2 genotypes were 2.5–3.4 μM lower than the predominant genotype, *GC*-1S2 (all pairwise comparisons $P < .001$, Supplemental Table 3). In contrast, *GC* genotype accounted for only 16% of the variation in pRID DBP, and although genotype was significantly associated with pRID DBP ($p_{ANOVA} < .001$), no genotypic group differed from another by more than

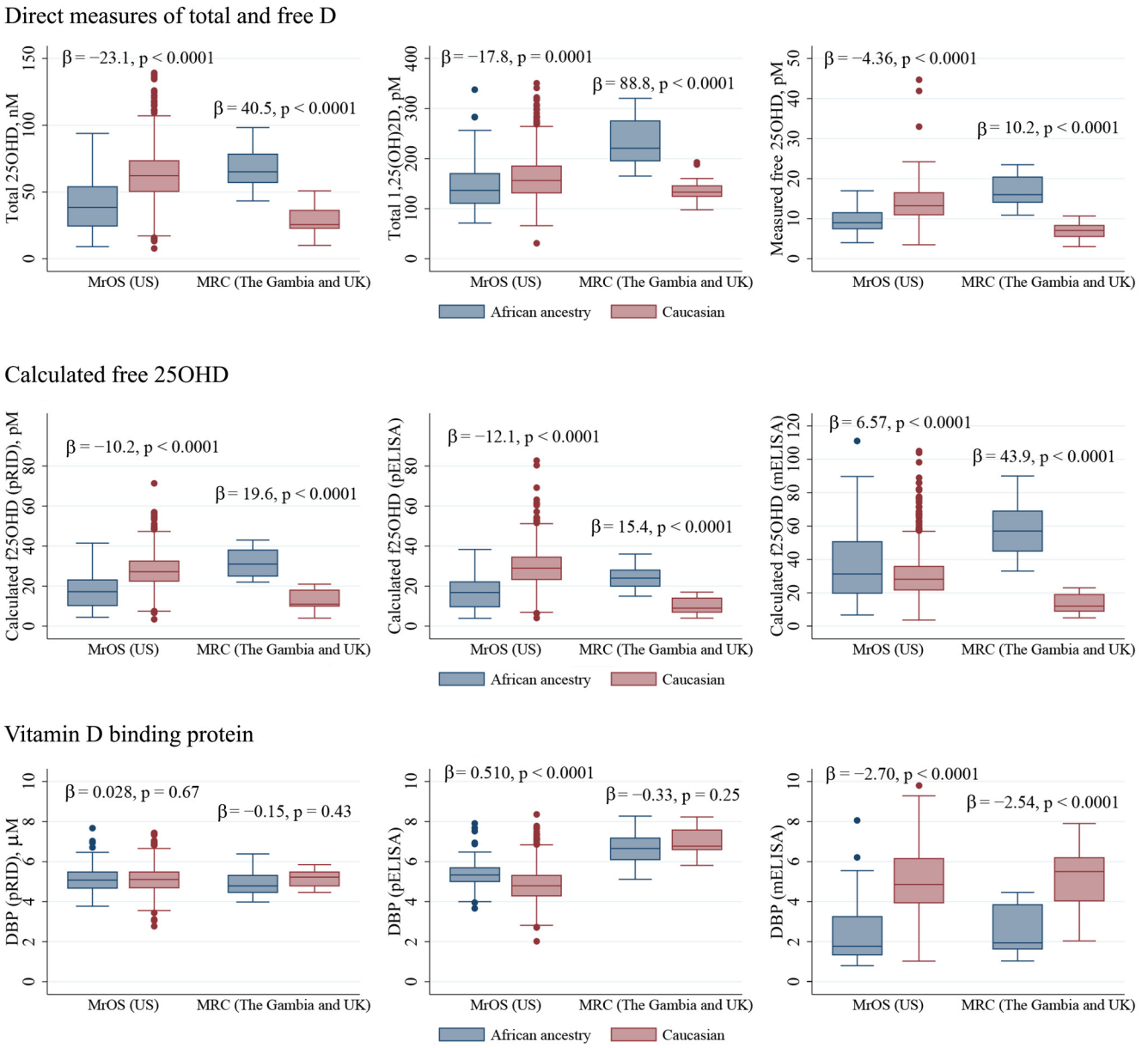

Figure 1. Total 25OHD, 1,25(OH)2D, free 25OHD, and vitamin D binding protein by race in MrOS and by racial-geographic group in MRC. β coefficients are the age- and BMI-adjusted difference in each measure for African-American men (n = 101) as compared to that of non-Hispanic white men (n = 919) in MrOS and for Gambian (n = 19) compared to UK (n = 18) men in MRC. Box and whisker plots show the 25th and 75th percentiles and median. Whiskers represent 1.5 times the interquartile range. Note differences in scale for free 25OHD measures resulting from the larger range of mELISA calculations and narrow range of directly measured free 25OHD. Comparisons were similar for calculated bioavailable 25OHD, which is strongly correlated with free 25OHD (r = 0.99). For measured free 25OHD in MrOS, African American n = 80 and non-Hispanic white n = 194.

0.59 μM. *GC* genotype had little influence on DBP measured by pELISA ($R^2 = 0.09$, $p_{ANOVA} = .02$, Figure 4, Supplemental Table 3).

SRM-based proteomic analyses of genotypically variant regions of DBP demonstrated that the amino acids predicted by the *GC* alleles were present in serum (Supplemental Figure 1). Moreover, peptides from genetically nonvariant regions were present in all samples and did not have lower abundance in men with a *GC*-1F allele (Figure 5). These results are consistent with DBP measures using pRID and pELISA, but not with mELISA.

Discussion

Irrespective of race, geographical region, or *GC* genotype, free 25OHD concentrations, both directly measured and calculated using polyclonal DBP measures, mirror total 25OHD concentrations. We found that African Americans consistently have lower free 25OHD and total 25OHD concentrations than US whites. We also showed that *GC* genotype determines the forms of circulating DBP peptides and strongly influences the measurement of DBP using a monoclonal ELISA. We posit that a unique sensitivity

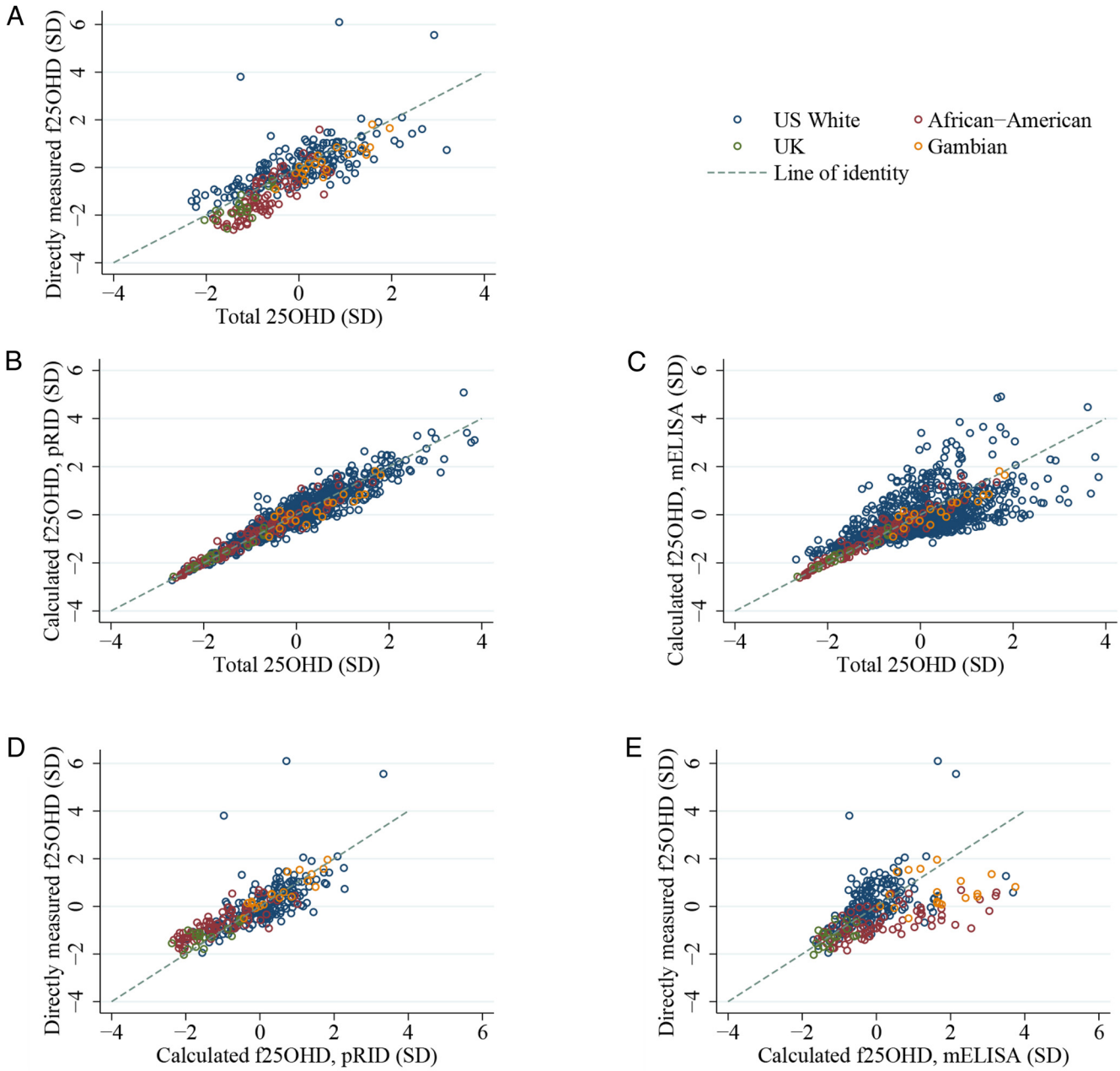

Figure 2. Relationships between total 25OHD and free 25OHD (A–C) and between measured and calculated free 25OHD (D, E). Calculations are the haplotype-constant estimates of free 25OHD, centered at the means and standardized (ie, each data point represents the distance from the mean for the participant). Results for pELISA are not shown but were similar to pRID.

to *GC*-1F DBP, common in those with African ancestry, underlies the low DBP concentrations assessed with that monoclonal ELISA, resulting in spuriously higher calculated free 25OHD and impeding the interpretation of racial comparisons. The results of several studies using the monoclonal DBP assay to calculate free 25OHD should therefore be reconsidered.

The recent reports (5, 13) of lower DBP concentrations in African Americans and consequently similar free 25OHD levels as in US whites despite a lower total 25OHD stimulated suggestions that concern for low 25OHD levels in African Americans is unfounded and that guidelines on vitamin D and public health policy should be revised according to race. Our results refute those findings and provide several internally consistent lines of evidence of a higher prevalence of vitamin D insufficiency in African Americans as based on both their free and total 25OHD. They indicate that the guidelines (1) concerning vitamin D and the assessment of vitamin D status on the basis of plasma 25OHD should be applied regardless of race. Although the current study did not address associations with clinical outcomes or whether lower 25OHD concentrations in African Americans might be related to important health consequences (eg, prostate cancer se-

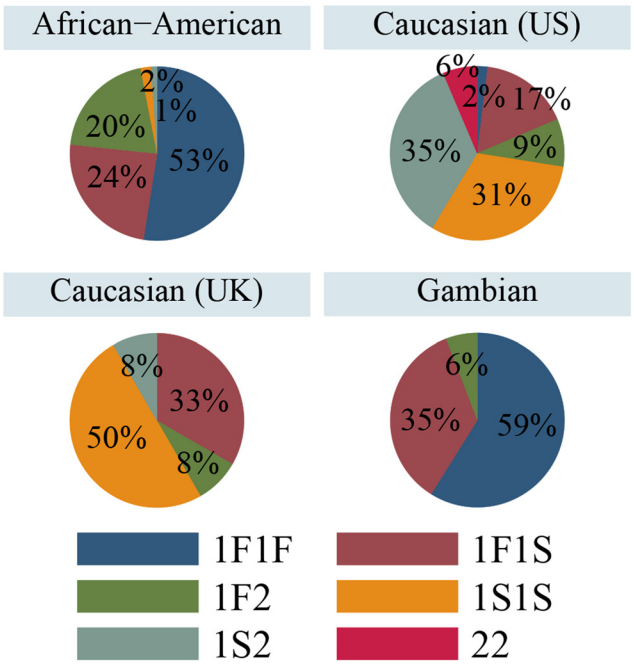

Figure 3. *GC* genotype by geographic-racial group.

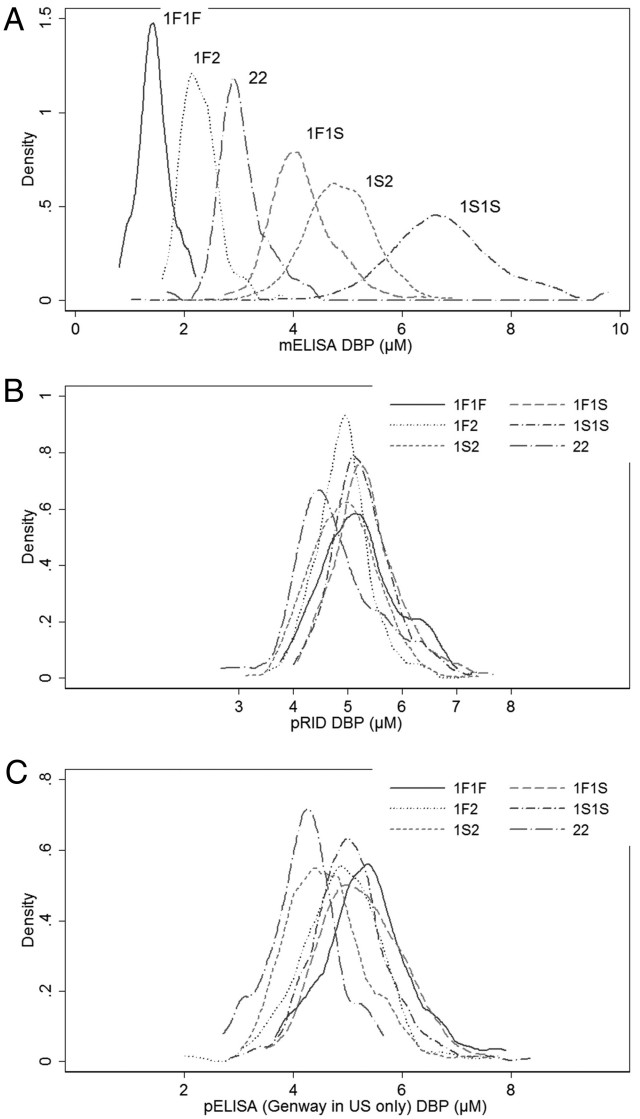

Figure 4. Circulating DBP concentrations by genotype, as assessed by mELISA (A), pRID (B), and pELISA (C). Genotype accounted for 83% of the variation in mELISA DBP and ≤16% for pRID and pELISA DBP. Concentrations of DBP differed by genotype (all $p_{ANOVA} < .001$), with *GC*-1F1F and 1F2 genotypes having the lowest values in the mELISA. In pRID and pELISA, *GC*-22 had significantly lower mean DBP than other genotypes (all pairwise comparisons provided in Supplemental Table 3).

verity (30) and cardiovascular mortality (31)), they do suggest that these remain critical research questions.

Previous studies that concluded that calculated bioavailable 25OHD in African Americans is similar to US whites despite differences in total 25OHD (13) depended on the derivation of free 25OHD using results from a monoclonal DBP. Here we show that this assay underestimates DBP concentrations in those with *GC*-1F alleles, a genotype more common in those of African ancestry and explains similar findings by others using the same monoclonal assay (5, 6, 13, 27, 32, 33). However, when DBP was assessed using immunoassays of DBP with polyclonal antibodies and proteomic methods these *GC*-dependent differences were not apparent, in line with other proteomic data (21). The lower detection of *GC*-1F DBP by mELISA, compared to relatively higher concentrations of nonvariant DBP peptides by SRM and higher concentration in polyclonal assays, can best be explained by the inadequate detection of *GC*-1F by the mELISA. Our direct measurements confirmed lower levels of free 25OHD in African Americans, and we found evidence that the active metabolite concentration and vitamin D activity is reduced (lower mean $1,25(OH)_2D$). Aloia et al reported that DBP concentrations measured by a polyclonal immunoassay were similar in US blacks and whites (33). However, directly measured free 25OHD was not significantly different between races, despite a lower total 25OHD concentration in African Americans. Although we cannot explain this discrepancy, we note that this is surprising; if the DBP concentration is similar in African Americans and whites, given a lower total 25OHD concentration, the law of mass action predicts that the free concentration of 25OHD will be proportionally lower. Here it was assumed that the association constant DBP-25OHD is the same across genotypes as suggested by most data (34–36). If the affinity of 25OHD for *GC*-1F is higher than for other genotypes as suggested by one study based on the binding affinity of a tracer for vitamin D rather than 25OHD (37), then the free 25OHD concentration would be even lower in African Americans.

We show that in African Americans, 25OHD concentrations (total and free) are lower than in US whites. In contrast, in Gambians, total and free 25OHD concentrations are higher than in UK whites. This cannot be

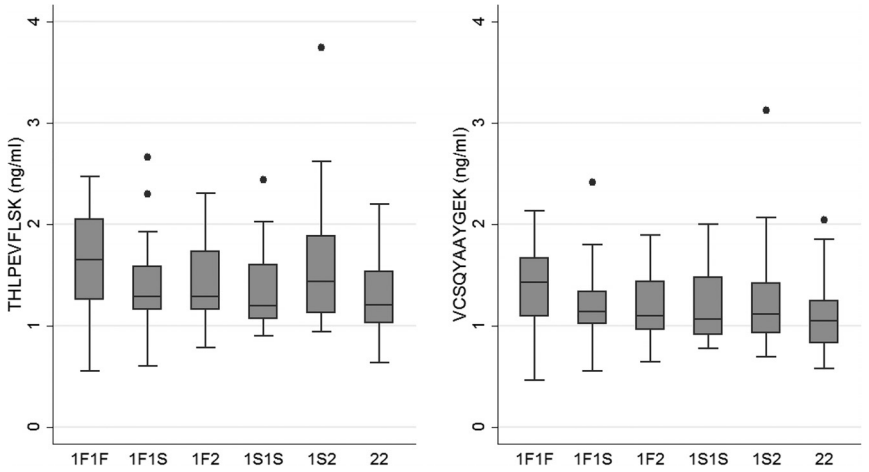

Figure 5. SRM results for two nonvariant peptides by *GC* genotype (n = 120, with 20 participants per GC genotype) The distribution of nonvariant peptides had similar concentrations across *GC* genotype. Participants with *GC*-22 had significantly lower concentrations than *GC*-1F1F ($P \leq .03$); however, no other genotype comparisons were statistically significantly different. Box and whisker plots show the 25th and 75th percentiles and median. Whiskers represent 1.5 times the interquartile range.

explained by racial differences in DBP concentrations, genotype, or affinity for 25OHD, but is consistent with the differences in total 25OHD between these groups, presumably the result of differences in their exposure to UV radiation and dermal production of vitamin D. Although our cohorts of Gambians and UK whites were small, our results mirror those from other African and UK studies (17, 38).

In our studies, calculated free 25OHD concentrations were higher than directly measured values; this may be due to lack of standardization of the direct measurements of free 25OHD and DBP or due to some uncertainty in the association constants used to calculate free 25OHD. However, the relative racial differences in free 25OHD were consistent for directly measured and calculated free 25OHD derived from DBP using polyclonal assays.

Although our study supports the comparability of total 25OHD with free 25OHD in terms of racial associations, there is need to improve the understanding of vitamin D metabolism through further evaluation of free 25OHD and other metabolites and their clinical relevance. Although free 25OHD represents less than 0.1% of the total 25OHD concentration, it may be more important for biological activity in most tissues. DBP undoubtedly has a central role in vitamin D biology; it may be involved in macrophage and osteocyte activation and may influence 25OHD concentration and metabolism (3, 19). There may be particular clinical value of free 25OHD measures in situations with large variation in circulating DBP concentrations (eg, pregnancy, estrogen use, liver or kidney disease) (9, 27). This idea is supported by knowledge that for some steroid hormones (eg, testosterone, thyroxine) measures of the free fraction may have more value than total levels (39). This question is particularly complex because gaps remain in our understanding of transport, storage, and distribution volume of the vitamin D metabolites; moreover the role of DBP in cellular uptake and subsequent metabolism and activity of vitamin D metabolites in different tissues remains to be further explored. Moreover, 25OHD is an intermediate metabolite with low affinity for the vitamin D receptor and reflects tissue availability of 25OHD for further hydroxylation into the more active metabolite, $1,25(OH)_2D$. Free $1,25(OH)_2D$ concentration is potentially a better reflection of its biological activity than total $1,25(OH)_2D$ concentration (10, 40).

Because calculated free 25OHD derives from measures of DBP, the accuracy of DBP assays in diverse populations is critical. Analyses by *GC* genotype suggest that the large racial differences in circulating DBP concentration are assay-dependent and related to *GC* genotype specific differences in performance of the monoclonal assay. This was confirmed by targeted proteomic analysis, showing that the concentrations of DBP peptides are not lower in individuals with *GC*-1F allele. Our results illustrate the importance of considering genetic variation in the accurate and complete determination of circulating protein concentrations.

In conclusion, previously reported calculations of higher bioavailable 25OHD in African Americans were influenced by *GC* genotype-dependent variations in DBP assay performance. In our studies, free 25OHD reflected total 25OHD, and the mean free 25OHD concentration was significantly lower in African Americans and UK whites than in US whites and Gambians, respectively, consistent with their mean total 25OHD concentration. Therefore, total 25OHD can be used in the general population as a marker of vitamin D status, irrespective of race or DBP genotype. In practice, clinicians should continue to measure total 25OHD in African Americans, and should continue to interpret low values, as defined by the Institute of Medicine, as an indication for supplementation.

Acknowledgments

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Author contributions: C.M.N. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. C.M.N., K.S.J., and Y.W. analyzed the data.

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